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14. ABSTRACT
The Six1 homeoprotein encodes a transcription factor that is critical during embryonic development. Six1 expression is normally limited to embryogenesis, however is found to be overexpressed in human breast cancers. Using cell culture and mouse models, we have previously shown that Six1 not only promotes proliferation andsurvival, contributing to tumorigenesis, but also upregulates the TGFβ pathway, brings about an EMT-like transformation, increases the cancer stem cell population, andpromotes metastasis. To further investigate the mechanism by which Six1 mediates the switch in TGFβ signaling, we performed a miRNA microarray screen and identified a cluster of miRNAs, the miR106b-25 cluster, that is upregulated in response to Six1 overexpression. The miR106b-25 cluster consists of three miRNAs, miR-106b, miR-93, and miR-25, which reside together in the intron of the MCM7 gene. Importantly, overexpression and knockdown experiments demonstrate that Six1 regulates all three miRNAs within the cluster. Interestingly, these miRNA have previously been implicated in the impairment of TGFβ-mediated growth suppression through repression of the cell cycle inhibitor, p21, and pro-apoptotic factor, Bim. These data suggest that Six1-induced upregulation of these miRNA may mediate the switch in TGFβ signaling from tumor suppressive to tumor promotional. Surprinsingly, bioinformatic analysis revealed that the miR106b-25 cluster may also contribute to the activation of TGFβ signaling through repression of the TGFβ signaling inhibitor, Smad7, which mediates the degradation of TβRI. Indeed, we now report that overexpression of the miR106b-25 cluster results in repression of the Smad7 3'UTR, with concominant upregulation of TβRI. Furthermore, activation of TGFβ signaling is observed with miR106b-25 overexpression,as demonstrated by an increase in phosphorylation of the downstream effector of the TGFβ pathway, Smad3, and by an upregulation of TGFβ transcriptional targets and alteration of the TGFβ response signature. Like Six1, the miR106b-25 cluster is sufficient to induce features of EMT, including a redistribution of E-cadherin, increased β-catenin transcriptional activation, upregulation of EMT related genes, and a decrease in cell matrix adhesion. Furthermore, the miR106b-25 cluster is also sufficient toincrease the cancer stem cell population as measured by in vivo serial dilution assays. In conclusion, we demonstrate that the Six1-mediated upregulation of miR106b-25allows for an altered response to TGFβ signaling in breast cancer cells, inhibiting the suppressive functions of TGFβ signaling while promoting the pro-metastatic functions.

15. SUBJECT TERMS
Six1, miRNAs, miR106b-25, TGF**β** , epithelial to mesenchymal transition (EMT), cancer stem cells, metastasis, Smad7

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INTRODUCTION:

The Six1 homeoprotein is an important regulator of embryonic development where it controls proliferation, survival, migration, invasion, and expansion of progenitor cell populations[2-4]. Six1 expression is normally restricted to embryogenesis, however Six1 is found to be re-expressed many cancers, including breast cancer, where it is overexpressed in 50% of primary cancers, and a striking 90% of metastatic lesions[5]. Using cell culture and mouse models, we and others have shown that Six1 contributes to tumorigenesis by not only promoting proliferation and survival[6], but also by inducing an EMT-like transformation and an increase in the cancer stem cell population[4,7], which has been linked to metastatic and invasive cancers[8]. We have demonstrated that Six1 upregulates TGFβ signaling, which is required for the Six1-induced EMT-like transformation and increase in cancer stem cell populations[7]. Interestingly, we have also demonstrated that Six1 is capable of switching TGFβ from a tumor suppressor to a tumor promoter[9], however the mechanism of this switch is still poorly understood. Currently, we are investigating miRNAs that may be involved in this Six1-induced metastatic process. Using miRNA microarray analysis and further validation studies we have identified a cluster of miRNAs that are regulated by Six1: the miR-106b-25 cluster. Located within the intron of the MCM7 gene, this cluster contains three miRNAs: miR-106b, miR-93, and miR-25. Previous research suggests that these miRNA have pro-oncogenic properties, as well as inhibiting the growth suppressive functions of TGFβ signaling[10,11]. Due to these findings, and other preliminary data in our lab, our aim in this proposal is to examine the Six1-upregulated miR106b-25 cluster and determine if these miRNA may be the mechanism by which Six1 switches TGFβ signaling from a tumor suppressor to a tumor promoter. In addition we also sought to determine if these miRNA alone are capable of activating TGFβ signaling, and if they are necessary and/or sufficient for Six1-induced metastatic phenotypes. The result of most of this work is in our now published manuscript in the journal Oncogene in 2012 (attached)[12]. Since this time, we have expanded on this work, and have continued research in the area of miR-106b-25 induced metastatic phenotypes.

BODY:

Aim1: To determine if the miR106b-25 cluster contributes to the activation of the TGFβ pathway by Six1 (month 1-7)

(A) Determine if the miR-106b-25 miRNAs target endogenous Smad6 and Smad7 (month 1-2)

This subaim is complete, and has been published in our recent manuscript[12]. From bioinformatic analysis we have identified that all three miRNAs in the miR106b-25 cluster have predicted binding sites in both the Smad6 and Smad7 gene. These genes are inhibitory Smads of the TGFβ pathway, therefore, by repressing these genes the miRNAs may play a role in activating TGFβ signaling[13]. Upon further analysis of the function of these genes, we decided to focus primarily on Smad7, since this gene is intimately involved in the TGFβ pathway, while Smad6 is primarily involved in BMP signaling[14]. Since we know that TGFβ signaling is critical for Six1-induced metastatic phenotypes, we have chosen to only look at Smad7 for this project. We have first shown that Smad7 mRNA is decreased in Six1 overexpressing cells (which is also present at

the protein level. When the 3'UTR of Smad7 was cloned into a luciferase reporter, we could also identify repression of this reporter (targeting of the 3'UTR) in MCF7 cells overexpressing the miR106b-25 cluster (MCF7-Cluster) versus an empty vector (MCF7-EV) and non-silencing control (MCF7-NS). Additionally, we've determined that Smad7 protein is also diminished in MCF7-Cluster cells by western blot analysis. Therefore, we have conclusively shown that the miR106b-25 cluster targets Smad7 for repression. Please see Figure 3 in our Oncogene manuscript for data related to this aim[12].

B) Determine if introduction of miR-106b-25 cluster miRNAs into wild type MCF7 cells can induce TGF-beta signaling (month 2-7)

This subaim has been completed, and is published in our recent manuscript[12]. To address this aim, we overexpressed the miR106b-25 cluster both transiently and stably into MCF7 cells. Upon overexpression we have demonstrated that TGFβ-receptor type I (TβRI) protein is upregulated with miR106b-25 overexpression (Fig4a-b in manuscript[12]). We have recently published data demonstrating that TβRI overexpression by Six1 is sufficient for TGFβ activation[9]. Therefore, we propose that the miR106b-25 miRNAs, at least in part, contributes to the upregulation of TβRI protein induced by Six1 overexpression. In addition, we also demonstrate an upregulation of phosphorylated-Smad3 (p-Smad3) in MCF7-Cluster cells, which is a molecular marker for activated TGFβ signaling (Fig4b in manuscript[12]). To strengthen this data, we have also recently added data showing that the miR106b-25 miRNAs are also sufficient to upregulate many genes that are downstream transcriptional targets of TGFβ signaling, as shown by quantitative real-time PCR. Thus, we have also now demonstrated that these miRNA can activate TGFβ signaling down to the transcriptional level (Fig4e in manuscript[12]).

Lastly, we have also conducted a microarray screen with our MCF7-NS and MCF7-Cluster cells in order to determine if the miR106b-25 cluster has any impact on the TGFβ response signature (TβRS) [1]. After analysis, we have shown that miR106b-25 overexpression has a differential response signature than the MCF-NS control cells (Supplementary Figure 7 in manuscript[12]). Our analysis shown in this figure is depicting only a 0.5 fold change, however, we believe that gene changes due to miRNA overexpression are not as strong as protein changes. Together, these data demonstrate that the miR106b-25 cluster can induce TGFβ signaling.

(C) Determine if knock down of miR-106b-25 miRNAs in MCF7-Six1 cells will reverse Six-1 induced increases in TGF-beta signaling (month 2-7)

This subaim has been completed, and has been published in our recent manuscript[12]. To address this aim, we looked at the same molecular markers as subaim B, which are TβRI protein and p-Smad3. From previous data in our lab, we know that Six1 overexpression will upregulate TβRI protein as well as increase the expression p-Smad3, both indications of increased TGFβ signaling. Therefore, to determine if the miRNAs will reverse Six1- induced increases in TGFβ signaling, we inhibited the miRNAs (individually, and together) in the MCF7-Six1 cell line. For miRNA inhibition, we used the miRzip lentiviral system from Open Biosystems. We prepared stable miRzip cell lines in our MCF7-Ctrl and MCF7-Six1 cell lines. For a control, we used a miRzip-Scramble construct, which does not inhibit any miRNA. Western blot of these cell lines demonstrates that inhibition of miR-93 and the entire cluster, will reverse the TβRI upregulation seen with Six1 overexpressing cells (Fig 4C in manuscript[12]). Additional western blots with these lysates indicate that knockdown of miR-106b, miR-93, as well as the entire Cluster, will reverse the p-Smad3 upregulation as seen with the Six1 overexpressing MCF7 cells with SCR control miRzip (MCF7-Six1-SCR) (Fig 4D in manuscript[12]). Therefore, based on these molecular markers for

TGFβ signaling, we conclude that we do, indeed, see a reversal of Six1- induced increased in TGFβ signaling.

Aim2: To determine if the Six1-regulated miR-106b-25 cluster miRNAs mediate the pro-metastatic EMT functions of TGF-beta while inhibiting its tumor suppressive functions (month 8-26)

(A) Determine if addition of miR-106b-25 miRNAs in wild type MCF7 will recapitulate the EMT phenotype of Six1 overexpression(month 8-12)

This subaim is complete and has been published in our recent manuscript[12]. The EMT phenotype as seen with Six1 overexpression includes a loss of E-cadherin and β-catenin from the membrane (insoluble fraction), and a gain of these proteins in the soluble fraction of the cell[7]. Additionally, we also report an increase in β-catenin transcriptional activity, via a Topflash reporter, as well as a decrease in adhesion to cell matrix proteins[7]. To address whether or not the miR106b-25 cluster can recapitulate these EMT phenotypes, we performed the same assays with MCF7-Cluster and MCF7-NS cells described above. Fractionation of the soluble and insoluble cell lysates shows a decrease of E-cadherin and β-catenin from the membrane (Insoluble fraction), however does not show an increase of these proteins in the soluble fraction (Figure 5a from manuscript[12]). Importantly, however, we also demonstrate that miR106b-25 cluster overexpression will increase β-catenin transcriptional activity as measured by the Topflash luciferase reporter (Fig 5b from manuscript[12]). In addition to these assays, we have also performed immunofluorescence with E-cadherin and β-catenin, and have demonstrated a loss of both of these proteins from the membrane of MCF7-Cluster cells (Fig5d from manuscript[12]). In addition to this we have also explored more genes involved in EMT in these cells by conducting an EMT gene PCR array (SABiosciences). Through this array, we have now reported that the miR106b-25 miRNAs will upregulate many EMT related genes in MCF7 cells (Fig5e). Lastly, we have completed an adhesion assay on our MCF7 cells, now with an additional control (MCF7-EV for empty vector control). These results now show that, like Six1, miR106b-25 overexpression will result in decreased adhesion to Collagen I, Collagen IV, and Fibronectin (Figure 5f from manuscrip[12]). These phenotypes suggest that the miR106b-25 cluster can induce many features of EMT, similar to Six1 overexpression in MCF7 cells.

(B) Determine if knock down of miR106b-25 miRNAs in MCF7-Six1 cells reverses the EMT induced by Six1 (month 12-16)

This subaim has been partially completed, and results represented here have been published in our recent manuscript[12]. To determine if the miR106b-25 cluster is necessary for Six1-induced EMT phenotypes, our plan was to do the same experiments as discussed in Aim2A, but now with an inhibition of the miRNAs in Six1 overexpressing cells. This task proved to be more difficult than miRNA overexpression since the technology for stable miRNA inhibition was not fully developed when we started this project. However, we were able to start experiments with transient oligonucleotide inhibitors. Namely, we were able to show that inhibition (via transient oligo inhibitors) of all three miRNAs together in MCF7-Six1 cells will decrease the Topflash activity back to MCF7-control levels (Figure 5c from manuscript[12]). Because this story has now been published, we are no longer working on doing anymore in vitro assays with EMT features, we have instead focused our efforts on our in vivo metastasis phenotypes, which provide more information on the *necessity* of the miRNAs than our in vitro EMT assays.

(C) Determine by an in vivo model, if the growth inhibitory effects of TGF-beta can be restored in MCF7-Six1 cells by knock down of miR-106b-25 cluster miRNAs (month 16-26)

This subaim has been partially completed. Interestingly, Six1 overexpressing cells exhibit a loss of TGFβ growth inhibition to which the mechanism is unknown. When cancer cells switch TGFβ signaling from a tumor suppressor to a tumor promoter, this is known as the TGFβ paradox. Previous research has described the miR106b-25 cluster as having the ability to switch TGFβ from a tumor suppressor to a tumor promoter via inhibition of the cell cycle inhibitor, p21, and the pro-apoptotic factor, Bim[11]. Therefore, we had speculated that the Six1-upregulated miR106b-25 cluster might be a mechanism by which Six1 overexpressing cells escape TGFβ growth inhibition (growth suppressive functions of TGFβ). To address this question, we had previous knowledge that MCF7 (as well as most cells in vitro) do not respond to TGFβ growth inhibition in vitro. However, we do know that we are able to see these effects in vivo. Therefore, we used and in vivo system to answer this question. Unfortunately, as mentioned in Aim2B, we originally had problems finding a stable knockdown of the miRNAs, so that we may use them *in vivo*. Therefore, we chose a system in which we could stably overexpress the miRNA, to answer the same question. Previous data in the lab has shown that when MCF7 cells are overexpressed with TβRI protein, and then injected in vivo, that these cells will turn on TGFβ signaling and have a growth suppressive effect versus the GFP control. Therefore, we decided to use our stable lentiviral miRNA overexpression constructs (miRexpress, Open Biosystems) in order to overexpress the miRNA in the context of TβRI overexpression. We speculated that if the miRNAs alone are able to overcome TGFβ growth inhibition, that we would see an increase in the tumor growth in MCF7-TBRI-Cluster cells

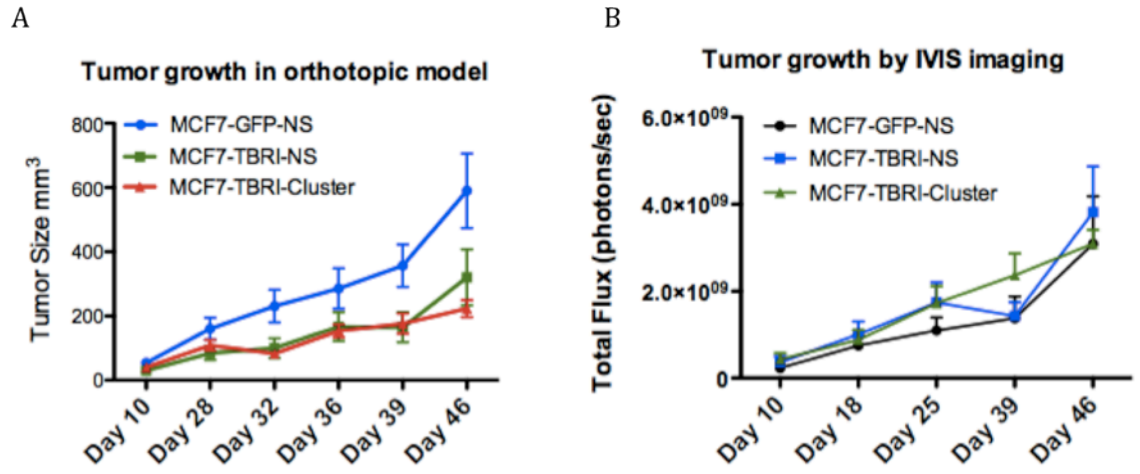
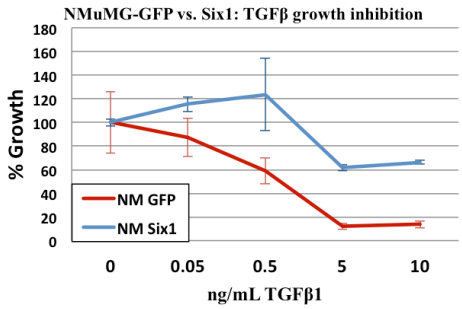


Figure 1: Cluster overexpression in MCF7-TBRI cells does not reverse TGFβ growth inhibition in vivo as measured by tumor growth (A) MCF7 cells overexpressing either GFP control or TBRI, and overexpressing NS control or the miR106b-25 cluster, were injected into the mammary fat pad of NOD-SCID mice. Tumor measurements over time are shown for each cell group by caliper measurement **(B)** same tumors as in (A) but now measured with IVIS imaging.

versus MCF7-TBRI-NS cells. In this experiment, we injected cells into the 4th mammary fat pad of NOD-SCID mice, and allowed tumors to grow. We measured tumor size over time using caliper measurements. Additionally, we also engineered these cells to have a luciferase reporter so that we may also measure tumor size by IVIS imaging, after delivering luciferase substrate to

the mice via IP injection. We used both methods to measure tumor volume over time, and unfortunately we did not see a reversal of tumor growth in the MCF7-TβRI-Cluster cells versus the MCF7-TβRI-NS cells as measured by a caliper (Fig 1A). The IVIS measurements, which are measuring the luciferase signal of the tumor cells, were inconclusive, as these measurements did not correlate with the actual tumor size (Fig 1B). We believe that the tumors in these mice were too large to measure by luciferase signal because they contained necrotic regions. This hypothesis was verified with tumor dissections to identify many necrotic regions in several of the tumors. From these experiments, we did not identify the miR106b-25 cluster as being sufficient for TGFβ growth inhibition in vivo. However, we believe that the upregulation of this cluster by Six1 may still be necessary for Six1's ability to evade TGFβ growth inhibition.



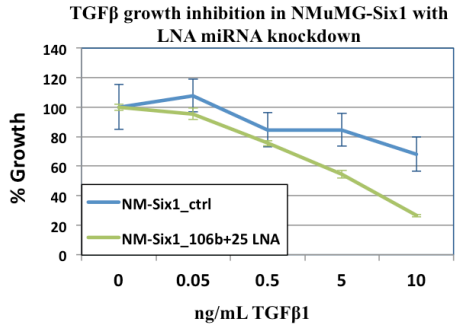


Figure 2. The miR-106b-25 miRNAs are required for Six1 to overcome TGF-β-mediated growth suppression. (a) Normal Murine Mammary Gland cells (NMuMG) overexpressing Six1 (NMuMG-Six1) or expressing a control plasmid (NMuMG-GFP) were subjected to increasing concentrations of TGF-β1 (added to the media). After 48 hours cells were assessed for their proliferative potential through 3H-Thymidine incorporation. Each cell line was plotted as % growth at individual concentrations of TGF-β1 as compared to vehicle treated cells with no TGF-β1 addition. NMuMG-GFP cells (red) exhibit a decrease in growth with TGF-β1 addition, while NMuMG-Six1 (blue) cells exhibit resistance to growth as compared to GFP control cells (b) miR-106b-25 cluster miRNAs were inhibited in NMuMG-Six1 cells with LNA-miRNA inhibitors. Inhibition of all three miRNAs + their family members (106b+25 LNA) in NMuMG-Six1 cells sensitized these cells to TGF-β1 growth inhibition, as read by MTS assay, and compared to NMuMG-Six1

Since our last report, we have additional evidence of the role of the miR106b-25 miRNAs in TGFβ growth suppression, and we have shown this by utilizing cell lines that do indeed respond to TGFβ in a growth suppressive manner, in culture. One of these cell lines is the normal murine mammary gland cell line (NMuMGs). In this system we have shown that when we overexpress Six1 in these cells (NMuMG-Six1), we have a decreased sensitivity to TGF-β growth inhibition as compared to control (NMuMG-Ctrl), as seen by a 3H-Thymidine incorporation assay with increasing TGF-β concentrations (Figure 2, top). In the NMuMG-Six1 cells, we have now utilized locked nucleic acid (LNA) inhibitors (provided by miRagen©) to knock down the miR106b-25 miRNAs in the context of Six1. When we knock down all three miRNA (miR-106b,93, and 25) in the NMuMG-Six1 cells, we observe a decreased sensitivity to TGF-β growth inhibition (Figure 2, bottom), suggesting that these miRNA do indeed play a role in Six1-mediated TGF-β growth suppression.

To strengthen this argument we have also identified a breast cancer cell line which contains both high levels of Six1 and the miR-106b-25 miRNAs, the BT549 cells. In a panel of breast cancer cell lines, these cell lines exhibit a higher expression of Six1/miR-106b-25 than other cell lines (Figure 3A). In this context, we have now also shown that knockdown of the miR-106b-25 miRNAs with LNA inhibitors also decreases the sensitivity of these cells to TGF-β growth inhibition (Figure 3B). Together, these additional in vitro results strengthen the hypothesis that

the miR-106b-25 miRNAs are necessary for Six1-mediated TGF-β growth suppression, and move us closer to understanding the mechanism of the TGF-β switch in the context of Six1 overexpression.

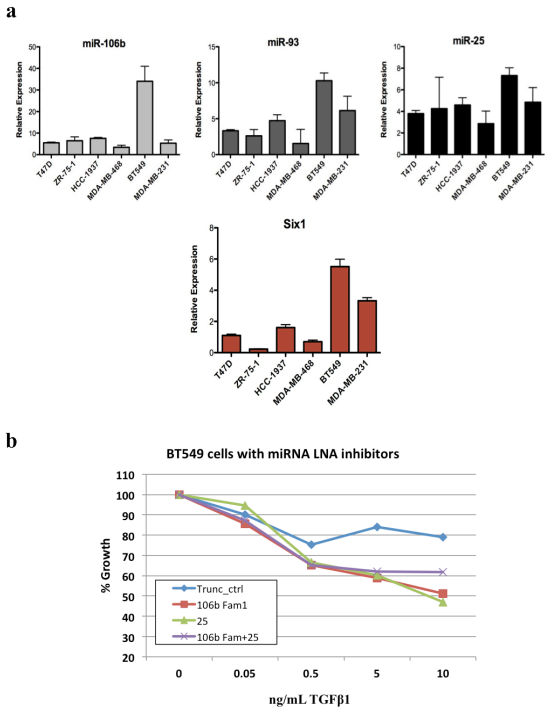


Figure 3. miR-106b-25 miRNA inhibition sensitizes BT549 cells to TGF-β growth inhibition. (a) All three miRNAs in the miR-106b-25 cluster have high endogenous expression in the BT549 cell line, as compared to other breast cancer cells (above), which corresponds to a high endogenous expression of Six1 in this particular cell line (bottom). qRT-PCR was performed for expression analysis, with normalization to U6 RNA for miRNA expression, and to PPIB for Six1 expression (b) BT549 cells with inhibition of miR-106b/93 (106b Fam1), miR-25 (25), or all together (106b Fam+25), exhibit decreased growth with increasing concentrations of TGF-β1, as measured by MTS assay, and compared to BT549 cells transfected with a control LNA (Trunc_Ctrl). Graph represents % growth, as measured in Figure B.1.

Aim3: To determine the in vivo role of the miR106b-25 cluster miRNAs in Six1-induced metastasis (month 26-36)

(A) Determine if miR106b-25 miRNA upregulation is sufficient to induce metastasis in an orthotopic xenograft breast cancer mouse model (month 26-31)

This subaim is mostly completed. To answer this question our first approach was to use an experimental metastasis model, in which we inject luciferase-taggeed cells into the left ventricle of the heart of nude mice, and follow metastasis of cells through IVIS imaging. We have previously published this metastasis model and the ability of MCF7-Six1 cells to increase metastasis over MCF7-Ctrl cells in this model[7]. Therefore, we decided to see if the MCF7-Cluster cells would increase metastasis over MCF7-NS cells in this model system, alongside MCF7-Ctrl and MCF7-Six1 cells to be able to compare the metastatic results. To achieve this, we luciferase-tagged our MCF7-NS and MCF7-Cluster cells, and used the MCF7-Ctrl and MCF7-Six1 luciferase tagged cells from the published experiment. We used all 4 cell lines and injected these cells intracardiac into nude

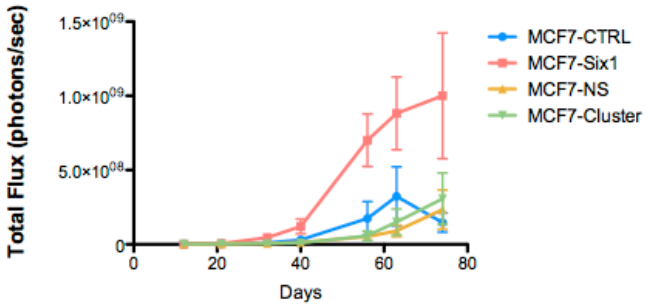


Figure 4: An experimental metastasis model does not show increased metastasis with Cluster overexpression. Luciferase tagged MCF7 cells overexpressing Ctrl, Six1, NS, and Cluster constructs were injected intracardiac into nude mice. Metastasis of these cells were measured by IVIS

mice (15 mice per group). We were able to measure metastasis via IVIS imaging over time (Fig 4). As before, we were able to see an increase in metastasis in the MCF7-Six1 cells over time, however, we did not see any increase in metastasis with MCF7-Cluster cells over their control cells (MCF7-NS) (Fig 4). The experimental metastasis model, however, is an assay designed to measure the late stages of metastasis. When cells are injected directly into the blood stream, the initial metastatic stages of intravasation, motility, and extravasation, are bypassed. Therefore, we cannot disclude the possibility that the miR-106b-25 cluster may be important for the earlier stages of metastasis.

We have, however, conducted orthotopic experiments for the purpose of discerning whether or not the miR-106b-25 cluster is sufficient to initiate tumors at limiting dilutions, which is a common assay used for cancer stem cell analysis. This assay did not include a metastasis element, but it did demonstrate that these miRNAs have an increased population of cancer stem cells, similar to Six1, as the mice that were injected with MCF7-Cluster cells had a significant increase in tumor formation over the NS control cells injected (Figure 6C in manuscript[12]). This assay not only demonstrates that the miR106b-25 cluster also increases the cancer stem cell population, but that we can demonstrate an increase in tumor formation in an orthotopic model.

(B) Determine if miR-106b-25 miRNAs are necessary for Six1-induced metastasis in an orthotopic xenograft breast cancer mouse model (month 31-36)

This subaim remains unanswered due to unforeseen complications. Since our last report, we have conduced an in vivo experiment to test the necessity of the miR-106b-25 miRNAs in Six1-mediated metastasis. Our approach, however, was not an orthotopic xenograft model. We chose to instead use our experimental metastasis model described above. Our reasons for this included the fact that this is a well established model system in our laboratory for Six1-mediated metastasis. In order to survey the necessity of the miR-106b-25 miRNAs in this system, we decided to take advantage of a collaboration with miRagen therapeutics, who has provided us with in vivo deliverable miRNA inhibitors. Thus, miRagen designed and manufactured specific LNA oligos that would inhibit the miR-106b-25 cluster miRNAs for our use *in vivo*. In our experimental design, we again injected MCF7-Ctrl and MCF7-Six1 (which contain high miR-106b-25 miRNAs) cells in the left ventricle of nude mice at Day0. Starting Day3, we treated our experimental group of animals with our LNA inhibitors through subcutaneous injections in the rear flank of the animals. Unfortunately, soon after LNA delivery, the animals developed a reaction to the LNA injections. Bruising and dark lesions occurred at the site of delivery (Figure 5). These unforeseen injuries lead to a disruption of LNA delivery, as our animal facility halted drug treatment in the second week of our experiment (LNA delivery originally planned bi-weekly). We continued the experiment in week3, and continued to treat the animals with LNA inhibitors and used IVIS imaging for monitoring metastasis. Our final results in metastasis did not reveal any significant changes with Six1-mediated metastasis in the

Figure 5. Unexpected lesions in mice as caused by miR-106b family LNA inhibitor subcutaneous injections. (Top) The picture reveals a nude mouse with a lesion on its left hind flank region at a 24-hour time point following LNA subcutaneous injection. (Bottom) Lesions continued to heal over time, and some lesions developed into tough scabbing around 1 week post LNA injection.

miR inhibited group (MCF7-Six1-106b+25 Fam) as compared to control Six1 overexpressing group (MCF7-Six1-SCR) (Figure 6). Although, we believe that disruption of LNA delivery in the second week of treatment could have been crucial in miR-induced colonization of metastasis. Therefore, we consider this aim incomplete, and plan to conduct this experiment differently, with genetic inhibition of miRs I cancer cells instead of systemic LNA miR inhibition.

Our results in the LNA experiment, however, bring about important questions concerning the possible side-effects of miR106b-25 inhibition. After pathological evaluation of our LNA specific lesions, we determined that the lesions were only caused by specific inhibition of the miR-106b/93 family of miRNAs. We also have determined that this was a skin specific lesion. Therefore, we believe that we have uncovered possible crucial information concerning miR-106b/93, and it's role in the skin. We believe this is an important avenue to follow-up with for future considerations of inhibiting these miRNA therapeutically.

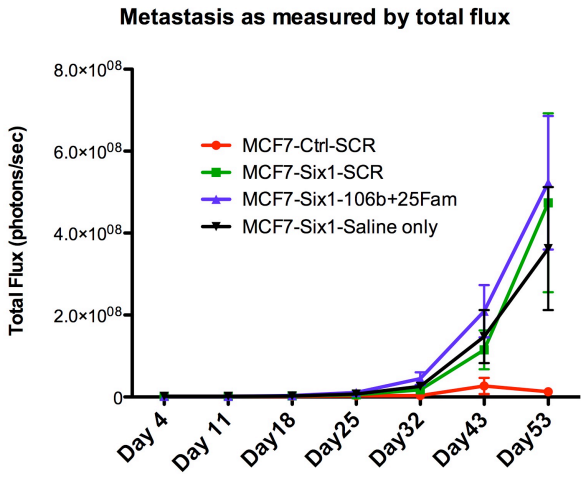


Figure 6. miR-106b-25 inhibition through LNA delivery reveals no difference in metastasis in MCF7-Six1 injected mice. In an experimental metastasis model, mice injected with MCF7-Ctrl or MCF7-Six1 cells were treated with LNA miR inhibitors that were either a scrambled ctrl (SCR), a combination of miR-106b family and miR-25 family inhibitors (106b+25Fam), or saline only. The graph represents total flux luciferase measurement in each animal group over time. Total flux values +/- standard deviation for each treatment group are shown at each time point. Data show no difference in metastatic burden of MCF7-Six1 mice treated with SCR, 106b+25Fam, or saline only.

Importantly, we do, however, have additional data suggesting that the miR-106b-25 miRNA are indeed necessary for Six1 metastatic phenotypes. We have yet to determine the actual impact on metastasis, however, we have discovered that the miR-106b-25 miRNAs are necessary for Six1-induced tumor initiation (cancer stem cell phenotype). We have demonstrated this in our latest manuscript by showing that MCF7-Six1 cells with miR-106b-25 knockdown (with stable miRZip lentiviral constructs) have a decreased ability to form tumors at limiting dilutions, as compared to control cells overexpressing Six1 (Figure 6D in manuscript[12]). Since the increase of cancer stem cells is an important property of metastasis, we consider this to be strong evidence that the miR-106b-25 miRNA, at least in part, contribute to Six1-induced metastasis.

Key Research Accomplishments: All accomplishments from start of funding are listed, accomplishments since last report are in bold type.

- Published many of these findings in a manuscript entitled: "The miR-106b-25 cluster targets Smad7, activates, TGF-beta signaling, and induces EMT and tumor initiating cell characteristics downstream of Six1 in human breast cancer" in the journal *Oncogene* in 2012.
- Determined that the miR106b-25 cluster targets the Smad7 gene for repression
- Determined that overexpression of the miR106b-25 cluster is sufficient to upregulate

TβRI protein, p-Smad3, and many TGFβ transcriptional targets. We also demonstrated a differential response in the TGFβ response signature, indicating activation of the TGFβ pathway by the miRNAs alone
- Identified that inhibition of miRNAs in Six1-overexpressing MCF7 cells will reverse the activation of TGFβ signaling as measured by decreased TβRI protein and p-Smad3
- Determined that overexpression of the miR106b-25 cluster alone will induce a loss of E-Cadherin and β-catenin from the membrane, as well as an increase in β-catenin transcriptional activity. We have also shown by immunofluorescence a loss of these proteins from the membrane of MCF7-Cluster cells. We have further shown that the miR106b-25 miRNAs induce EMT through an induction of many EMT related genes and a reduction of adhesion to cell matrix proteins. Thus, conclusively indicating that these miRNAs are sufficient to induce features of EMT.
- Determined that knock down of the miR106b-25 cluster is sufficient to reverse β-catenin transcriptional activity in Six1 overexpressing MCF7 cells
- Identified that the miR106b-25 cluster alone is not sufficient to increase metastasis in an experimental metastasis model (late stage metastasis)
- Determined that the miR106b-25 miRNAs are sufficient AND necessary for tumor initiation (or cancer stem cell capacity) in an *in vivo* tumor initiating assay.
- **Now have further evidence in support of the necessity of the miR-106b-25 miRNAs in Six1-mediated TGF-β growth inhibition**
- **Have determined that miR-106b/93 inhibition leads to adverse side-effects in the skin of nude mice, which is a possible new avenue for a role for these miRNA in the skin.**

Reportable outcomes for 2013:

First author review based on TGF-β biology:

Smith AL, Robin TP, & Ford HL. Molecular Pathways: Targeting the TGF-β pathway for Cancer Therapy. *Clin Cancer Res.* **18**, 4514-4521 (2012)

Research presented in the form of a poster:

Smith AL, Iwanaga R, Drasin DJ, Micalizzi DS, Vartuli RL, Ford HL. The miR-106b-25 Cluster as a Potential Target for Six1-mediated Breast Cancer Metastasis, and a Molecular Marker of the TGFβ Paradox. EORTC-NCI-AACR Molecular Targets and Cancer Therapeutics, *Dublin, Ireland, November 6-9, 2012* See appendix for poster abstract.
- Bolie graduate scholarship fund travel award (from Molecular Biology Program at UCDenver AMC) awarded for travel to this conference

Research presented in the form of a thesis defense:
- March 1st, 2013: "The role of the miR-106b-25 miRNAs in Six1-mediated breast cancer progression and metastasis"
- phD degree in Molecular Biology obtained
- post-doctoral positions offered based research in this project (no decisions made yet)

Conclusions:

The results from studies up to this point have shown that the miRNAs (miR-106b, miR-93, and miR-25) all have an impact on Six1 induced tumorigenesis. We have demonstrated that the miR106b-25 cluster can repress the TGFβ inhibitor Smad7, thus having an effect on activation of the TGFβ pathway. Indeed, we have demonstrated that the miR106b-25 cluster is both sufficient and necessary for activation of TGFβ signaling. We have conclusively shown that these miRNA are sufficient for EMT induction, similar to Six1, through a loss of both E-cadherin and β-catenin from the membrane, a loss of cell matrix adhesion, and an upregulation of several genes involved in EMT. This work has also lead to our increased understanding of a role for the miR-106b-25 miRNAs in cancer stem cell biology. We have now demonstrated that the miR-106b-25 miRNAs are both necessary and sufficient for Six1-induced cancer stem cell phenotypes. We do no not yet know if these phenotypes are through the miR-induced increase in TGF-β signaling. Importantly, we have made continued progress this year in the attempts to determine the role of the miRs in Six1-induced TGFβ growth suppression. We now have further evidence in two additional cell lines confirming that the miR-106b-25 miRNAs are important for the TGF-β switch downstream of Six1.

We were not able to demonstrate that the miR106b-25 cluster is sufficient for increased metastasis through an experimental metastasis model. However, it is possible that these miRNAs may only be necessary and not sufficient for metastasis. When attempting to determine the necessity of the miRs in Six1-induced metastasis, we uncovered a possible side-effect of miRNA inhibition using LNA miRNA inhibitors delivered sub-cutaneously. We believe that this disturbance caused our results in this experiment to be interpretable, and therefore we plan to conduct this experiment again, but with genetic downdown of the miRNAs instead of systemic inhibition with LNAs. Since we have shown that these miRNA are important for TGFβ signaling, features of EMT, as well as cancer stem cell phenotypes, we anticipate that we will see an effect on Six1-induced metastasis when inhibiting the miRNAs through a different mechanism.

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Appendices:

Poster abstract for EORTC-NCI-AACR Molecular Targets and Cancer Therapeutics, Dublin, Ireland, November 6-9, 2012:

The miR-106b-25 Cluster as a potential target for Six1-mediated breast cancer metastasis, and a molecular marker of the TGFβ paradox.

Previous work in our laboratory has demonstrated a critical role for the homeoprotein Six1 in breast cancer metastasis. Our data has shown that Six1 controls metastatic spread through an upregulation of TGFβ signaling, leading to an EMT-like transformation and an increase in the tumor initiating cell (TIC) population. TGFβ is a multifunctional cytokine, which can function as either a tumor suppressor or a tumor promoter, making this pathway a difficult target in cancer therapy. Additionally, our data shows that the presence of Six1 in cells will implement the switch of TGFβ from tumor suppressive to tumor promotional.

To further understand the mechanism by which Six1 mediates metastasis through TGFβ signaling, we explored miRNAs that may be involved in this process. Through a miRNA microarray and further validation studies, we found that Six1 upregulates a cluster of three miRNAs, the miR-106b-25 cluster, which is comprised of miR-106b, miR-93, and miR25. Interestingly, this cluster has already been implicated in the impairment of TGFβ growth suppression through repression of the cell cycle inhibitor, p21, and pro-apoptotic factor, Bim. Therefore suggesting that upregulation of these miRNA by Six1 may help mediate the switch in TGFβ signaling. Indeed, preliminary results in NMuMG (normal mouse mammary gland) cells and BT549 breast cancer cells demonstrates that inhibition of the miR-106b-25 miRNAs in the context of Six1 overexpression restores TGFβ-mediated growth inhibition. In addition to its role in TGFβ growth suppression, we have also recently identified that this same cluster of miRNA is

capable of activating the tumor promotional functions of TGFβ. We have shown that these miRNA bind and repress the TGFβ inhibitor, Smad7, and activate downstream TGFβ signaling through upregulation of p-Smad3 and TGFβ transcriptional targets. Upon further exploration, we also find that the miR-106b-25 cluster is sufficient to induce EMT-like features as well as an increase in the TIC population, and that these miRNA are required downstream of Six1 to induce these phenotypes. Lastly, we also demonstrate a significant correlation of Six1 and miR-106b expression in human breast cancers, and further show that high expression of miR-106b or miR-93 correlates to shortened time to relapse in breast cancer patients. All together, we find that the Six1-mediated increase in the miR-106b-25 cluster may not only be involved in facilitating the oncogenic switch of TGFβ, but also aids in the activation of this pathway, thus contributing to the pro-metastatic phenotypes found with Six1 overexpression. Because it is difficult to target transcription factors like Six1 directly, our data points to a new therapeutic target for Six1 expressing breast cancers (miR-106b-25), which may also be used to predict which patients should benefit, rather than be harmed, by TGFβ inhibitors which are currently in clinical trials.

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ORIGINAL ARTICLE

The miR-106b-25 cluster targets Smad7, activates TGF-β signaling, and induces EMT and tumor initiating cell characteristics downstream of Six1 in human breast cancer

AL Smith[1], R Iwanaga[2], DJ Drasin[1], DS Micalizzi[1,3], RL Vartuli[1], A-C Tan[4] and HL Ford[1,2,3,5]

The role of TGF-β signaling in tumorigenesis is paradoxical: it can be tumor suppressive or tumor promotional, depending on context. The metastatic regulator, Six1, was recently shown to mediate this switch, providing a novel means to explain this elusive 'TGF-β paradox'. Herein, we identify a mechanism by which Six1 activates the tumor promotional arm of TGF-β signaling, via its ability to upregulate the miR-106b-25 microRNA cluster, and further identify a novel function for this cluster of microRNAs. Although expression of the miR-106b-25 cluster is known to overcome TGF-β-mediated growth suppression via targeting p21 and BIM, we demonstrate for the first time that this same cluster can additionally target the inhibitory Smad7 protein, resulting in increased levels of the TGF-β type I receptor and downstream *activation* of TGF-β signaling. We further show that the miR-106b-25 cluster is sufficient to induce an epithelial-to-mesenchymal transition and a tumor initiating cell phenotype, and that it is required downstream of Six1 to induce these phenotypes. Finally, we demonstrate a significant correlation between miR-106b, Six1, and activated TGF-β signaling in human breast cancers, and further show that high levels of miR-106b and miR-93 in breast tumors significantly predicts shortened time to relapse. These findings expand the spectrum of oncogenic functions of miR-106b-25, and may provide a novel molecular explanation, through the Six1 regulated miR-106b-25 cluster, by which TGF-β signaling shifts from tumor suppressive to tumor promoting.

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Keywords: Six1; miRNA; TGF-β; epithelial-to-mesenchymal transition; tumor-initiating cell

INTRODUCTION

TGF-β signaling has a critical and dual role in breast tumorigenesis. In normal epithelium as well as in early tumorigenic lesions, TGF-β has a tumor suppressive role through its ability to induce growth inhibition. As cancer progresses, however, tumor cells become resistant to TGF-β-mediated growth inhibition, and instead TGF-β promotes tumor progression and metastasis, likely in part through its promotion of an epithelial-to-mesenchymal transition (EMT).[1,2] The ability of TGF-β to switch cells from tumor suppressive to tumor promotional has been coined the TGF-β paradox.[3] The mechanism of this phenomenon is not well understood, but remains an important area of research.

Recently, our laboratory identified the developmental homeotic transcription factor, Six1, as an important mediator of breast cancer progression and metastasis.[4–7] We have found that Six1 is dependent on upregulation of TGF-β signaling to induce an EMT,[7] an increase in tumor-initiating cell (TIC) characteristics[8] (and data not shown), and to induce late stage metastasis.[7] Interestingly, using an experimental metastasis model, we demonstrated that Six1 not only upregulates TGF-β signaling, but that it also switches the pathway from tumor suppressive to tumor promotional.[7,9] However, until now, the mechanism by which Six1 accomplishes this switch was unknown.

Herein, we have identified a cluster of miRNAs, miR-106b-25, that is upregulated by the homeoprotein Six1. This cluster is highly conserved in vertebrates, and consists of three miRNAs, miR-106b, miR-93 and miR-25, which all reside in the 13th intron of the *MCM7* gene (Chr7). Two paralogs of this cluster exist, the miR-17-92 cluster (Chr13, *c13ORF25* gene), and the miR-106a-363 cluster (ChrX). The miR-17-92 cluster has received considerable attention as a pro-oncogenic cluster, and was the first cluster of miRNA identified to cooperatively act as an oncogene.[10] Interestingly, the miR-106b-25 cluster of miRNA is overexpressed in several cancers,[11–15] and like the miR-17-92 paralog, is pro-oncogenic.[15] Previous reports have demonstrated the ability of these miRNA to inhibit the growth suppressive functions of TGF-β signaling through repression of downstream mediators p21 and Bim.[11] In this study, we show for the first time that this cluster of miRNA can target the TGF-β inhibitor Smad-7 and also *activate* the TGF-β-signaling pathway, providing a novel mechanism by which Six1 overexpression can mediate the switch in TGF-β signaling from tumor suppressive to tumor promotional. We also demonstrate that overexpression of the miR-106b-25 miRNAs is sufficient to induce characteristics of EMT and TICs, and that this cluster is necessary for the ability of Six1 to mediate these phenotypes. Finally, we demonstrate a significant correlation between miR-106b expression and both Six1 and activated TGF-β signaling in human breast cancer tissues, and further show that high expression levels of miR-106b and miR-93 together in early-invasive breast tumors can significantly predict a shorter time to

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relapse for these patients. Taken together, our results significantly expand the tumorigenic functions of the miR-106b-25 cluster. In addition, we demonstrate a critical role for this cluster in mediating not only the pro-tumorigenic functions of Six1, but also provide a possible mechanism by which Six1 overcomes TGF-β-mediated growth suppression, whereas simultaneously activating the pro-metastatic arm of the pathway.

RESULTS

Six1 regulates the miR-106b-25 Cluster of miRNAs

Previous studies have demonstrated substantial cross-talk between miRNAs and homeobox genes.[16,17] We therefore asked whether the Six1 homeoprotein might regulate miRNAs to mediate its tumorigenic and metastatic phenotypes. miRNA microarray analysis on RNA isolated from MCF7 breast cancer cells overexpressing Six1 (MCF7-Six1) and control cells (MCF7-Ctrl) led to the identification of several miRNAs that were differentially expressed in a statistically significant manner between the two groups (Figure 1a). Interestingly, we identified two miRNAs, miR-106b and miR-25, that were upregulated in response to Six1 overexpression (Figure 1a), and that belong to a cluster of miRNAs, which also includes miR-93, and reside in the 13th intron of the *MCM7* gene (Figure 1b). These miRNA have previously been implicated as a pro-oncogenic cluster of miRNAs.[12,15,18] To validate our microarray results, we performed quantitative real-time reverse transcriptase PCR (qRT–PCR) on an independent set of RNA isolated from MCF7-Ctrl and MCF7-Six1 cells, demonstrating that all three miRNA within the cluster are overexpressed 2–3 fold in MCF7-Six1 cells as compared with MCF7-Ctrl cells (Figure 2a). In addition, siRNA knockdown of Six1 in 21PT cells (Supplementary Figure 1), which contain high levels of Six1 endogenously,[6] resulted in a clear decrease in all three miRNAs, confirming that endogenous Six1 regulates the miR-106b-25 cluster (Figure 2b). Finally, to examine whether Six1 could regulate the miR-106b-25 cluster *in vivo*, we analyzed expression of the miRNA cluster in transgenic mice in which Six1 was induced (using doxycycline) in the mammary gland (Six1 + Dox) and in control animals (Ctrl + Dox)[8] (See Supplementary Figure 2 for expression levels of Six1 in the transgenic mammary glands) and found that all three miRNAs, miR-106b, miR-93 and miR-25, are overexpressed in Six1 transgenic mammary glands as compared with control mammary glands (Figure 2c). Importantly, Six1 transgenic mice develop aggressive mammary carcinomas that display multiple histological subtypes as well as an induction of an EMT.[8]

The miR-106b-25 Cluster targets Smad7 for repression

It was previously shown that the miR-106b-25 cluster has the ability to overcome TGF-β-mediated growth suppression via repression of the cell cycle inhibitor p21, and the pro-apoptotic factor Bim.[11] In addition to the known role of the cluster in TGF-β growth inhibition, using target prediction analysis, we found that the miR-106b-25 cluster might also have a role in *activating* the TGF-β pathway, providing an attractive mechanism by which Six1 could mediate the switch in TGF-β signaling from tumor suppressive to tumor promoting. Indeed, based on seed sequence alignment, the inhibitory Smad7 (I-Smad7) mRNA is a target for all three miRNAs in the cluster. Smad7 antagonizes TGF-β signaling through multiple mechanisms, including binding to TGF-β type I receptor (TβRI) and interfering with recruitment and downstream phosphorylation and activation of the receptor-Smads, Smad2 and Smad3.[19] Additionally, Smad7 also functions to recruit E3 ubiquitin ligases to TβRI, resulting in its degradation.[20] Therefore, repression of Smad7 by the miR-106b-25 miRNAs would be expected to activate the TGF-β-signaling pathway, which is known to occur downstream of Six1.[7]

To determine if Smad7 is downregulated in response to Six1, we first performed qRT–PCR on clonal isolates of MCF7-Ctrl and MCF7-Six1 cells and demonstrated that Smad7 expression is indeed reduced in MCF7-Six1 cells, where the miR-106b-25 cluster is overexpressed (Figures 3a and d shows protein level differences). To further determine whether the cluster of miRNAs can directly affect Smad7 levels, we generated MCF7 cell lines stably overexpressing the genomic region of the cluster (MCF7-Cluster), or control MCF7 cells expressing either the empty vector (MCF7-EV) or a non-silencing (scrambled control) vector (MCF7-NS). Importantly, stable populations expressing the cluster were chosen to overexpress each miRNA in the cluster only 2 to 3-fold, similar to what is observed with Six1 overexpression (Supplementary Figure 3). Transfection of a Smad7-3′UTR-luciferase construct into these cell lines demonstrates that the miR-106b-25 cluster inhibits the 3′UTR of Smad7 (Figure 3b). Additionally, a decrease in Smad7 protein in MCF7-Cluster cells is observed when compared with MCF7-EV and MCF7-NS cells, demonstrating that a 2–3 fold

a miRNA microarray

MCF7-Ctrl vs. MCF7-Six1	Fold Change	*P*-Value
hsa-miR-106b	**+1.2**	**0.0137**
hsa-miR-1234	+1.2	0.0468
hsa-miR-219-1-3p	+1.1	0.0410
hsa-miR-25	**+1.3**	**0.0468**
hsa-miR-181a	-1.9	5.73E-06
hsa-miR-181b	-1.4	8.00E-05
has-miR-622	-1.8	5.90E-07
hsa-miR-191	-1.1	0.0073
hsa-miR-652	-1.3	0.0138
hsa-let7b	-1.3	0.0468
hsa-miR-939	-1.3	0.0138
hsa-miR-423-3p	-1.3	0.0138
hsa-miR-500*	-1.2	0.0305

b

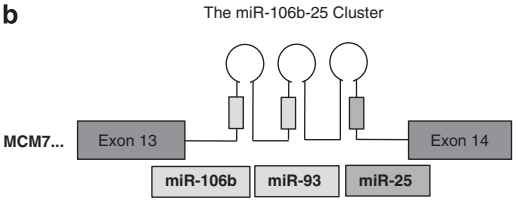

Figure 1. A miRNA microarray identifies the miR-106b-25 cluster family members as upregulated by Six1. (**a**) miRNAs that are significantly up- or downregulated (*P* value <0.05) in MCF7-Six1 vs MCF7-Ctrl cells as determined by a miRNA profiling array. (**b**) Schematic representation of the miR-106b-25 cluster of miRNA (miR-106b, miR-93 and miR-25) within the 13th intron of the *MCM7* gene.

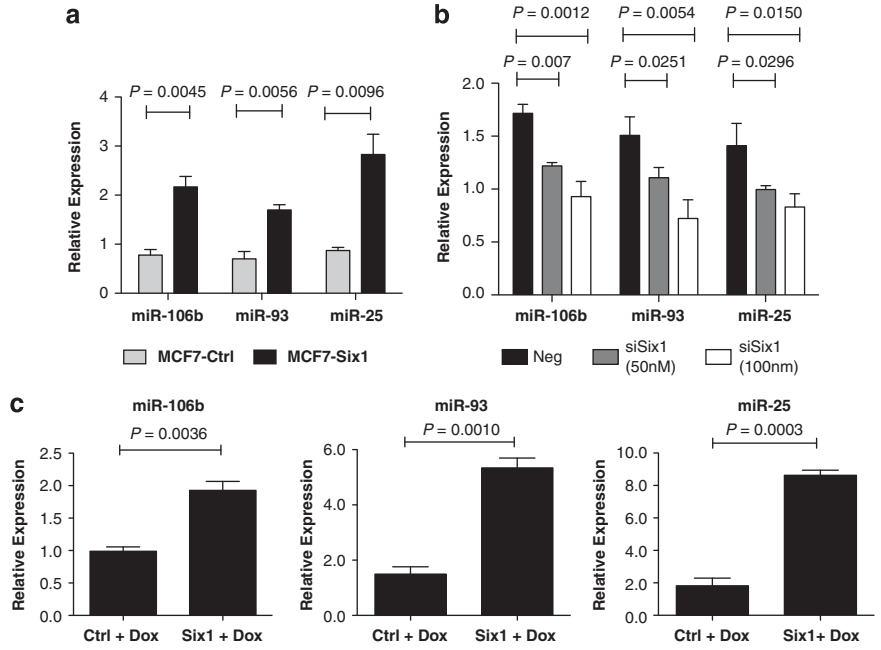

Figure 2. Six1 regulates the miR-106b-25 Cluster. (**a**) Stable overexpresson of Six1 in MCF7 cells leads to an increase in miR-106b, miR-93 and miR-25 as determined using qRT-PCR. Data are represented as the mean ± s.e.m. of three individual MCF7-Six1 and MCF7-Ctrl clones. (**b**) Knockdown of Six1 in 21PT cells using Six1-specific siRNA (siSix1, 50 nm and 100 nm) leads to a decrease in expression of all three miRNA in the miR-106b-25 Cluster when compared with a control knockdown (siNeg). For qRT-PCR analysis, the average of three replicates ± s.d. is shown. (**c**) RNA was isolated from the mammary glands of bitransgenic mice in which Six1 was induced with doxycycline (Six1 + Dox) versus single transgenic MTB control mice also treated with Dox (Ctrl + Dox), but unable to express Six1. qRT-PCR performed on the isolated RNA for the miR-106b-25 miRNAs demonstrates an increase in expression of all three miRNAs in the Six1 + Dox mammary glands, which express high levels of the Six1 transgene (Supplementary Figure 2) as compared with Ctrl + Dox control mammary glands. $n = 3$ mice for each condition, and each miRNA was normalized to U6 RNA. *P* values represent statistical analysis using a paired *t*-test.

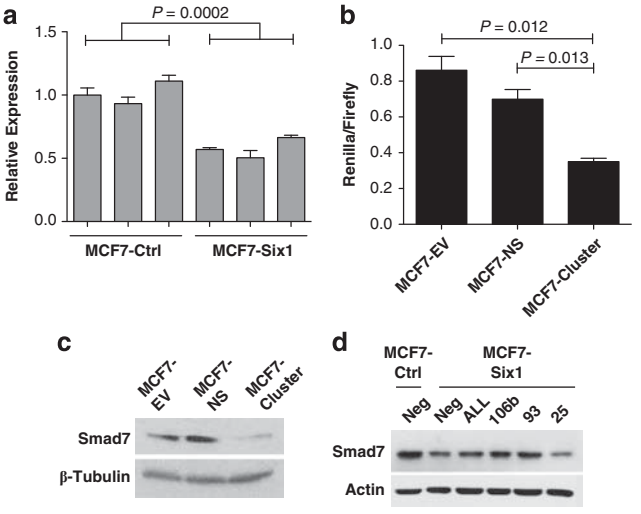

Figure 3. The miR-106b-25 miRNAs repress Smad7. (**a**) qRT-PCR reveals a decrease in Smad7 mRNA in MCF7-Six1 cells versus MCF7-Ctrl cells. Data shown are the result of three replicate qRT-PCR reactions (± s.d.), normalized to cyclophilin B mRNA levels. (**b**) A renilla luciferase reporter containing the 3′UTR of Smad7 was transfected into MCF7 cells containing an EV (MCF7-EV), a NS control (MCF7-NS), or the miR-106b-25 cluster (MCF7-Cluster). Measurement of renilla luciferase normalized to firefly luciferase (present on the same vector but expressed from a different promoter) demonstrates a significant repression of the Smad7 3′UTR in response to miR-106b-25 expression. (**c**) Western blot analysis demonstrates that MCF7-Cluster cells have decreased Smad7 protein as compared with MCF7-EV and MCF7-NS cells. (**d**) MCF7-Ctrl and MCF7-Six1 cells treated with miRNA inhibitors towards all three miRNA (ALL), miR-106b and miR-93, show a de-repression of Smad7 protein in MCF7-Six1 cells. *P* values represent statistical analysis using a paired *t*-test.

increase in the miR-106b-25 cluster can downregulate endogenous Smad7 (Figure 3c). Conversely, treatment of MCF7-Six1 cells with transient inhibitors against the individual miRNAs leads to a de-repression of Smad7 protein, with miR-106b and miR-93 being the major mediators of this effect (Figure 3d). Efficacy of the miRNA inhibitors is demonstrated by relative activity of luciferase reporters, containing target sites for each miRNA, in the inhibitor transfected cells (Supplementary Figure 4).

The miR-106b-25 Cluster activates TGF-β signaling

Because the miR-106b-25 cluster represses Smad7 in MCF7 cells, and because Six1 overexpression, which leads to increased levels of the miR-106b-25 miRNAs, activates TGF-β signaling,[7] we asked whether this cluster of miRNAs, which is known to overcome TGF-β-mediated growth inhibition, is also sufficient to activate the TGF-β pathway. Indeed, with both transient and stable overexpression of the miR-106b-25 cluster, we observed an increase in TβRI protein levels (Figures 4a and b), as well as an increase in activated TGF-β signaling as measured by p-Smad3 levels (Figure 4b). To further determine if the miR-106b-25 miRNAs are necessary for the previously observed induction of TGF-β signaling by Six1, we utilized a stable lentiviral miRNA knockdown system (miRZip) to inhibit the miRNAs within the cluster either individually or together (as a control, we used a scrambled sequence, miRZip-SCR). Efficacy of the miRZips was demonstrated by examining their effects on endogenous targets of the miR-106b-25 cluster, p21 and BIM (Supplementary Figure 5). Inhibition of miR-93, as well as the entire cluster in MCF7-Six1 cells reverses the Six1-induced increase in TβRI (Figure 4c), and inhibition of miR-106b, miR-93, as well as the entire cluster reverses the Six1-induced increase in p-Smad3 (Figure 4d).

Interestingly, previous reports have identified the miR-106b-25 cluster miRNAs as targeting the TGF-β type II receptor (TβRII),

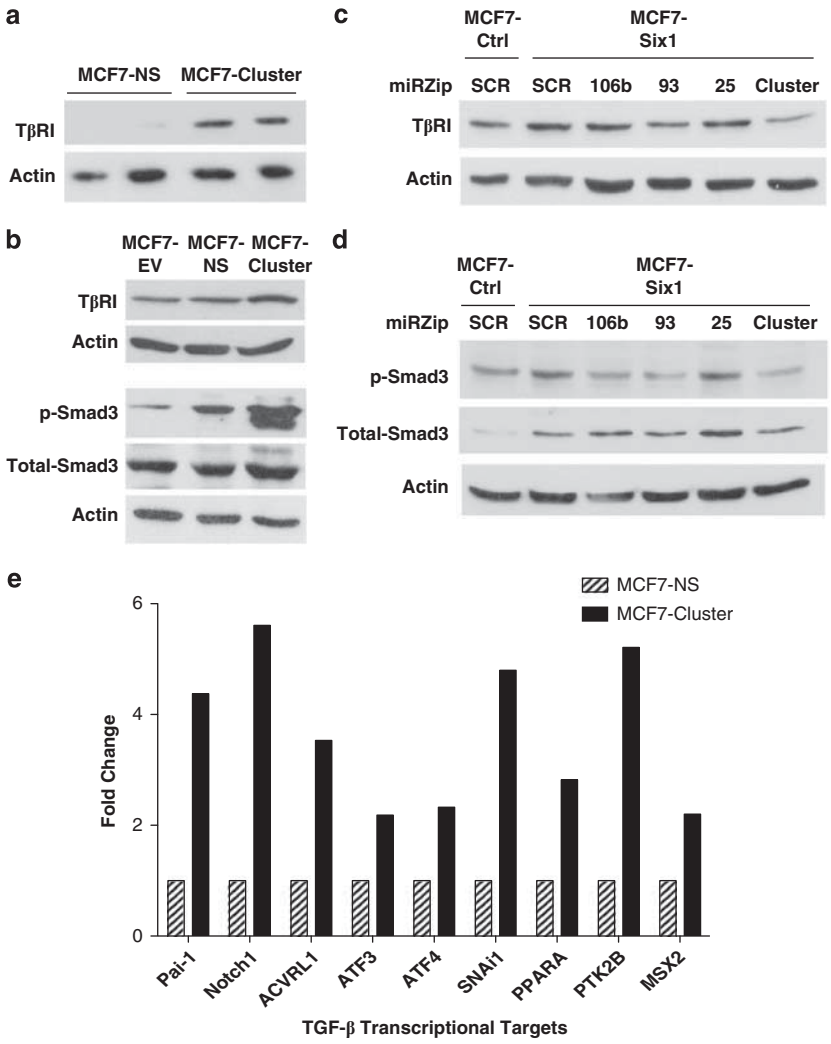

Figure 4. The miR-106b-25 cluster activates TGFβ signaling. (**a**) Transient and (**b**) stable overexpression of the miR-106b-25 cluster in MCF7 cells leads to increased expression of TβRI protein over controls and an increase in phosphorylated Smad3 (p-Smad3). (**c**) MCF7-Ctrl and MCF7-Six1 cells expressing stable miRZip inhibitors targeting the miR-106b-25 miRNAs individually and together (miRZip-Cluster) show a reversal of the Six1-induced increase in TβRI protein in miRzip-93 and miRZip-Cluster treated MCF7-Six1 cells as compared with scramble miRZip controls (miRZip-SCR). (**d**) Introduction of miRZip-106b, miRZip-93 and miRZip-Cluster into MCF7-Six1 cells reverses the Six1-induced increase in p-Smad3 levels, without affecting the Six1-induced increase in total Smad3 levels. (**e**) A real-time PCR array containing TGF-β transcriptional targets shows enrichment for TGF-β target gene expression in MCF7-Cluster cells over MCF7-NS cells. Data are represented as fold change expression of MCF7-Cluster compared with control MCF7-NS from three replicate plates of each condition. Genes shown had at least a twofold induction in MCF7-Cluster cells as compared with MCF7-NS cells.

resulting in repression of this protein.[21] Analysis of TβRII protein in our MCF7-Cluster cells did not show a repression of TβRII protein as compared with MCF7-NS cells (Supplementary Figure 6). Similarly, we also did not observe significant downregulation of TβRII protein levels in MCF7-Six1 versus MCF7-Ctrl cells (Supplementary Figure 6). Thus, 2–3 fold overexpression of the miR-106b-25 cluster in MCF7 cells leads primarily to alterations in the TGF-β pathway that would be expected to be activating, as opposed to inactivating.

To analyze global changes in TGF-β signaling, we performed microarray analysis on MCF7-NS versus MCF7-Cluster cells to examine whether the presence of the miR-106b-25 cluster alters the TGF-β response signature (TβRS),[22] similar to what is observed with Six1 overexpression.[7] Hierarchical clustering confirmed differential regulation of many of the genes in the TGF-β response signature between MCF7-NS and MCF7-Cluster cells, demonstrating that TGF-β signaling is indeed altered in response to miR-106b-25 overexpression (Supplementary Figure 7). In addition, we performed a qRT–PCR array to examine alterations in expression of TGF-β target genes using MCF7-NS and MCF7-Cluster cells, demonstrating that TGF-β signaling is clearly activated downstream of the miR-106b-25 cluster, as numerous TGF-β transcriptional targets are upregulated in MCF7 cells overexpressing the cluster (Figure 4e). Of the 84 genes responsive to TGF-β signaling on the array, 47 were upregulated 1.5 fold or more in MCF7-Cluster cells, 8 genes were downregulated, and the rest remained unchanged (Supplementary Figure 8). Together, these data demonstrate that the miR-106b-25 cluster is capable of activating TGF-β signaling in breast cancer cells, and suggest that this one cluster, which can also overcome the growth suppressive effects of TGF-β, may be responsible for the switch in TGF-β signaling from tumor suppressive to tumor promoting.

The miR-106b-25 cluster induces EMT-like changes

We previously reported that Six1 overexpression leads to an induction of EMT, which is dependent on TGF-β signaling.[7]

As the miR-106b-25 cluster is sufficient to activate TGF-β signaling, we asked if this cluster is sufficient to mediate phenotypes associated with EMT. One of the hallmarks of EMT is the loss of membranous E-cadherin from the adherens junctions. We thus analyzed the subcellular localization of E-cadherin from each cell line and demonstrated that E-cadherin is indeed decreased in the insoluble, or membrane bound, fraction of the cell in response to miR-106b-25 overexpression (Figure 5a). β-Catenin, which is normally in the membranous adherens junctions with E-cadherin, is also decreased in the insoluble fraction of MCF7-Cluster cells (Figure 5a). As redistribution of β-catenin away from the membrane may result in its increased nuclear localization and subsequent ability to activate transcription, we measured β-catenin transcriptional activity using the TOP-flash luciferase reporter. Concomitant with the loss of β-catenin from the membrane, MCF7-Cluster cells also exhibit an increase in TOP-flash reporter activity over MCF7-NS cells, similar to the phenotype observed with Six1 overexpression (Figure 5b). To determine if the miR-106b-25 miRNAs are necessary for the Six1-induced increases in β-catenin transcriptional activation, we treated MCF7-Six1 cells with inhibitors toward all three miRNA (miR-106b, miR-93 and miR-25) together and measured TOP-flash activity. A repression of TOP-flash activity in this context demonstrates that Six1 is dependent on the miR-106b-25 miRNAs to induce β-catenin transcriptional activity (Figure 5c). Furthermore, the relocalization of E-cadherin and β-catenin away from the membrane in response to miR-106b-25 cluster expression was confirmed using immunofluorescence (Figure 5d).

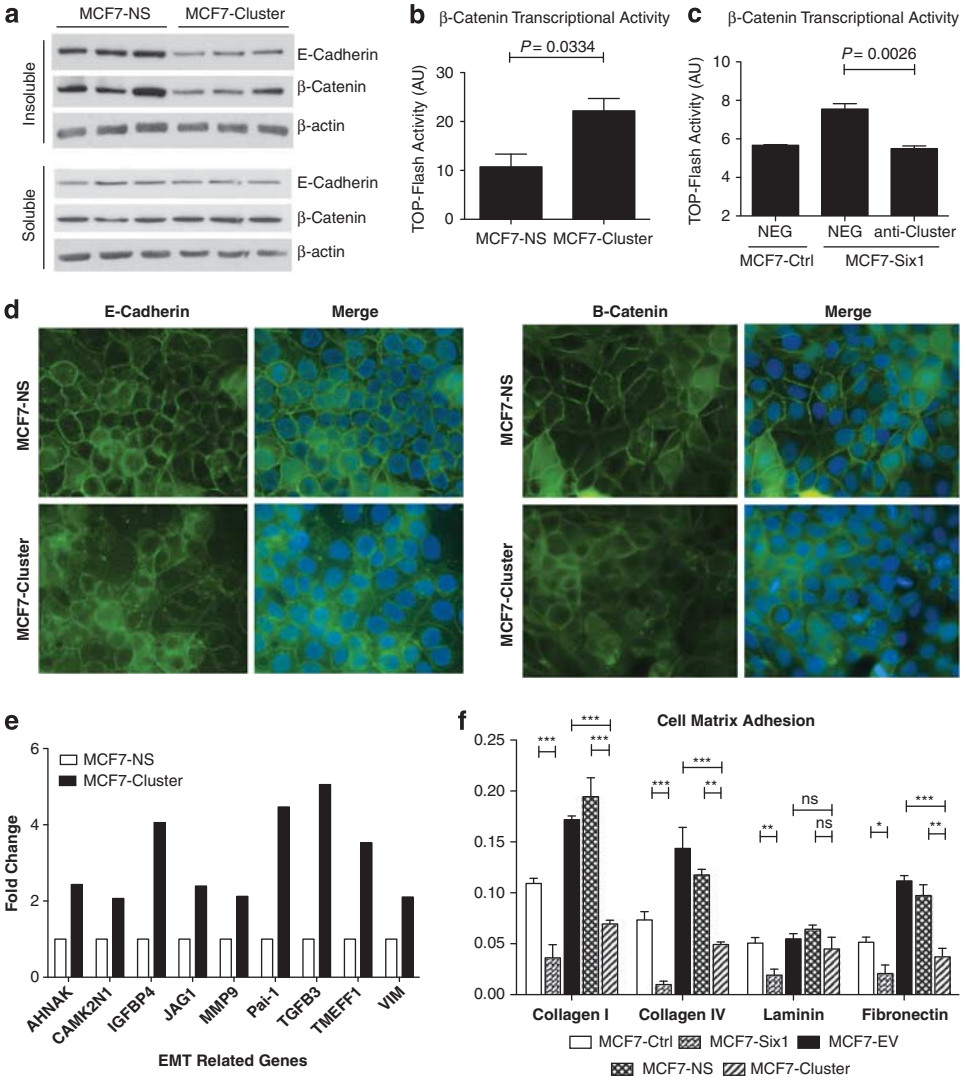

Figure 5. The miR-106b-25 cluster mediates features of EMT (**a**) miR-106b-25 overexpression results in loss of E-cadherin and β-catenin from the insoluble (cytoskeleton-associated) protein fraction of the cell as determined by western blot analysis. (**b**) MCF7-Cluster cells show increased activity of the β-catenin responsive-luciferase reporter TOP-Flash, normalized to Renilla luciferase activity. Data are from three replicates ± s.d. (**c**) The Six1-induced increase in TOP-Flash activity is reversed with inhibition of the miR-106b-25 cluster using transient hairpin inhibitors (Dharmacon, miRidian, Lafayette, CO, USA). (**d**) Immunofluorescence to detect E-cadherin and β-catenin in MCF7-NS vs MCF7-Cluster cells. After incubation with the E-cadherin (left panel) or β-catenin (right panel) antibodies, a FITC conjugated anti-secondary antibody was added to detect the signal. Dapi images merged with FITC are shown along with the FITC images (× 40 magnification). (**e**) Expression of genes involved in EMT is enriched in MCF7-Cluster cells versus MCF7-NS cells as shown by a qPCR array analysis. Gene expression is shown by fold change from three replicate plates per group. Genes shown exhibited a twofold or greater induction in MCF7-Cluster cells when compared with MCF7-NS cells. (**f**) Expression of the miR-106b-25 Cluster in MCF7 cells results in decreased adhesion to cell matrix proteins Collagen I, Collagen IV and Fibronectin, similar to what is observed with Six1 overexpression. *P* values represent statistical analysis using a paired *t*-test (* ⩽ 0.05, ** ⩽ 0.01, *** ⩽ 0.001).

To determine whether additional genes associated with EMT are differentially regulated between MCF7-NS and MCF7-Cluster cells, we performed qRT–PCR array analysis on RNA isolated from each cell line. Indeed, miR-106b-25 expression led to a 2-fold or greater upregulation of a number of genes associated with EMT, including Jag1, MMP9 and Vimentin, amongst others (Figure 5e, Supplementary Figure 9 shows all genes regulated 1.5-fold or more). Finally, we observed a decrease in cell–matrix adhesion to Collagen I, Collagen IV and Fibronectin in the MCF7-Cluster cells as compared with the MCF7-NS and MCF7-EV cells, similar to the decrease in adhesion observed with Six1 overexpression in MCF7 cells (Figure 5f). Together, these data suggest that the miR-106b-25 cluster alone can induce an EMT-like phenotype.

The miR-106b-25 cluster increases tumor-initiating cell characteristics

Many genes that induce EMT-like phenotypes also induce TIC phenotypes.[23] Indeed, we recently demonstrated that Six1 induces a TIC phenotype in both a transgenic mouse model[8] and when overexpressed in MCF7 cells.[24] To determine if the miR-106b-25 miRNAs, which can induce properties of EMT, can also induce TIC characteristics, we performed flow cytometry for the cell surface TIC-associated markers CD24 and CD44,[25] and found that miR-106b-25 did indeed increase the percentage of CD24low CD44 + cells, similar to Six1 (Figure 6a). Additionally, secondary tumorsphere assays performed with MCF7-Cluster and MCF7-NS cells demonstrated that, similar to MCF7-Six1 cells,

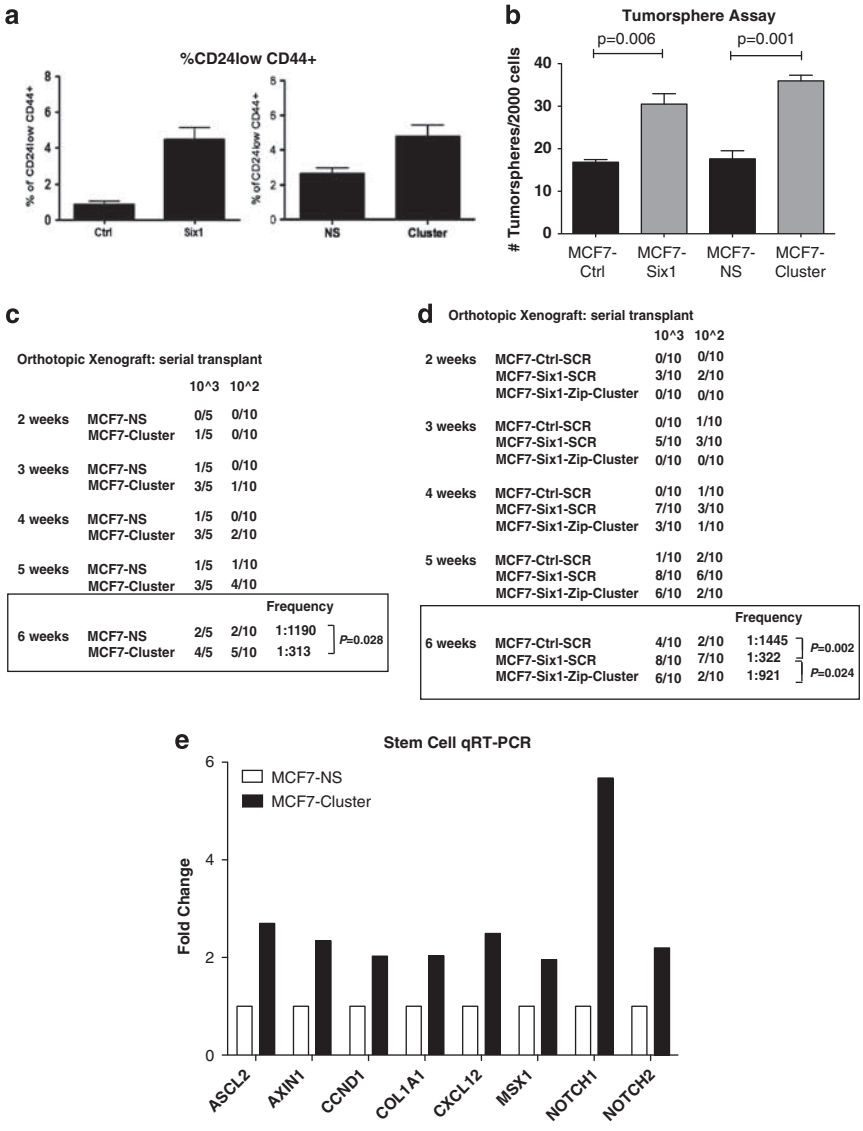

Figure 6. The miR-106b-25 cluster increases TIC characteristics. (**a**) Overexpression of the miR-106b-25 cluster in MCF7 cells is sufficient to increase the CD24low/CD44 + population, similar to what is observed with Six1 overexpression in MCF7 cells. (**b**) Expression of the miR-106b-25 cluster in MCF7 cells is sufficient to increase tumorsphere formation, a measurement of self-renewal capability, similar to what is observed with Six1 overexpression in MCF7 cells. (**c**) MCF7-Cluster cells transplanted into the 4th mammary fat pad of NOD-SCID mice at limiting dilutions have an increased ability to initiate tumors when compared with MCF7-NS cells. (**d**) Inhibition of the miR-106b-25 cluster in MCF7-Six1 cells (MCF7-Six1-Zip-Cluster) reduces the ability of the cells to initiate tumors, back to levels observed in MCF7-Ctrl cells (MCF7-Ctrl-Zip-SCR). The estimated frequency of tumorigenic cells was calculated by limiting-dilution analysis as described in the methods. (**e**) Genes important for stem cell maintenance, growth and differentiation are increased in MCF7-Cluster cells as compared with MCF7-NS cells, as determined by a stem cell qPCR array. Data are represented as fold change in gene expression in MCF7-Cluster cells as compared with control MCF7-NS cells from three replicate plates of each condition. The Genes shown exhibited at least a twofold induction in MCF7-Cluster cells as compared with the MCF7-NS cells.

MCF7-Cluster cells could increase tumorsphere formation, a measure of functional TICs within a population (Figure 6b). To further test for functional TIC characteristics using an *in vivo* assay, we injected cells at limiting dilutions into the mammary fat pad of NOD-SCID mice. MCF7-Cluster cells were able to initiate tumors with a greater frequency than MCF7-NS cells, both when 1000 and 100 cells were injected (Figure 6c). In order to determine if the miR-106b-25 cluster was necessary for Six1-induced increases in TICs *in vivo*, we utilized our MCF7-Six1-miRZip-Cluster cells (in which all three miRNAs are inhibited), and transplanted these cells into the mammary gland at limiting dilutions, along with miRZip-SCR controls in both MCF7-Ctrl and MCF7-Six1 cells. Inhibition of the miR-106b-25 miRNAs in MCF7-Six1 cells demonstrates a reduction in tumor initiating ability back to similar levels of TIC frequency seen in MCF7-Ctrl cells (Figure 6d). Lastly, we performed a human stem cell qRT–PCR array. Of the 84 genes on this array, 53 were upregulated more than 1.5 fold in MCF7-Cluster cells (Figure 6e and Supplementary Figure 10). Together these data demonstrate for the first time a role for the miR-106b-25 cluster in both EMT and in increased TIC capacity.

The miR-106b-25 cluster correlates with Six, activated TGF-β signaling, and shortened time to relapse in human breast cancer

To determine if the Six1/miR-106b-25/activated TGF-β signaling axis is relevant to human breast cancer, we obtained human breast cancer tissue arrays containing 71 cases of invasive ductal carcinoma, with matched cases on which we had previously performed immunohistochemistry using an antibody generated against human Six1 and human Smad3 (nuclear Smad3 was scored as an indicator of activated TGF-β signaling).[7] We next performed *in situ* hybridization for miR-106b (as a representative of the miR-106b-25 cluster), to compare expression of this cluster family member with nuclear staining obtained with the Six1 antibody and with activated TGF-β signaling. Importantly, miR-106b and Six1 significantly correlate in breast cancer tissues ($P = 0.0028$ Spearman's $R = 0.3927$) (Figure 7a), as do miR-106b and nuclear Smad3 ($P = 0.0017$, Spearman's $R = 0.3972$) (Figure 7b). In addition, the greatest percentage of tumors exhibited activated TGF-β signaling when both miR-106b and Six1 were highly expressed (64.7% show increased nuclear Smad3 when both miR-106b and Six1 are high) (Figure 7c). Of note,

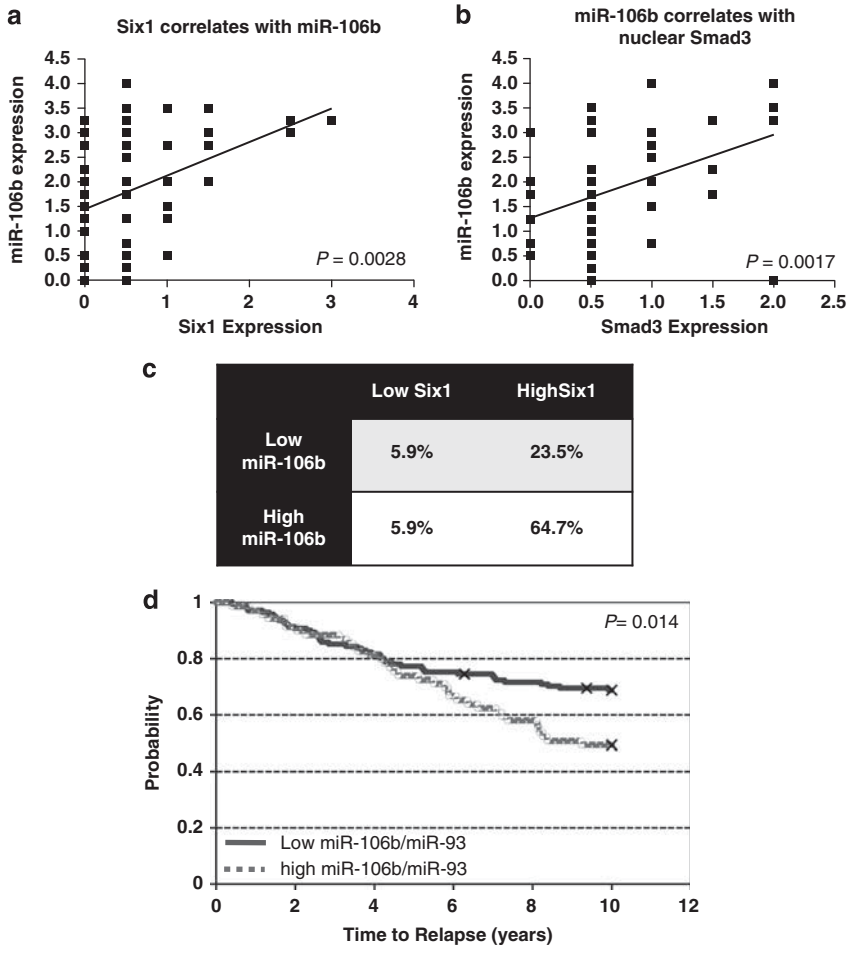

Figure 7. The miR-106b-25 cluster correlates with Six1 expression, activated TGFβ signaling and shortened time to relapse in human breast cancers. Human breast cancer tissue arrays were previously immunostained with an anti-Six1 antibody (Atlas) and a Smad3 antibody (Zymed) as previously described[7] and the staining was scored for nuclear Six1 and Smad3 on a scale of 0–4. A serial section array was also stained for miR-106b expression by *in situ* hybridization. Expression of miR-106b was scored on a scale of 0–4 and compared with Six1 and nuclear Smad3 scores in the same tissues. (**a**) Results show that miR-106b and Six1 correlate in human breast cancers (**b**) as do miR-106b and nuclear Smad3. (**c**) When the expression of all three molecules are considered, the highest percentage of nuclear Smad3 can be found when both Six1 and miR-106b are highly expressed. *P*-values obtained using Spearman's correlation analysis. (**d**) In a miRNA expression dataset of 216 early-invasive breast cancers,[27] patients whose tumors express both high miR-106b and high miR-93 show a significantly reduced time to relapse. The median value for miR-106b and miR-93 was used to divide the samples into high (above median) and low (below median) miRNA expression. *P* value was calculated by log-rank analysis. A full colour version of this figure is available at the *Oncogene* journal online.

the Six1 antibody used in these experiments was generated against a region of Six1 that may allow cross-reactivity with other Six family members, as they are highly conserved. Therefore, we can only confidently say that Six family expression correlates with miR-106b in human tumors. However, examination of the van de vijver public breast cancer gene expression dataset[26] suggests that Six1 is more abundantly expressed than other Six family members in human breast tumors (data not shown). Together, these data strongly suggest a critical role for miR-106b-25 in the Six1-induced activation of TGF-β signaling in human breast cancer.

To explore the prognostic value of these miRNA in human breast cancers, we examined a publicly available dataset comprised of miRNA expression in early-invasive breast cancers.[27] Figure 7d demonstrates that patients whose tumors express high miR-106b and high miR-93 together have a significantly shortened time to relapse. Analysis of individual miRNA expression in these tumors also demonstrates a significant correlation with high miR-93 (Supplementary Figure 11B), as well as a trend toward shortened time to relapse with high miR-106b and high expression of all three miRNA (Supplementary Figure 11A,D). However, miR-25 expression does NOT demonstrate any difference in patient outcome (Supplementary figure 11C), further suggesting that miR-106b and miR-93 are the primary regulators of this response.

To further explore the role of miR-106b and miR-93 in human breast cancers, we also utilized gene set enrichment analysis (GSEA) to analyze breast tumors that express high levels of miR-106b and miR-93. Of the enriched gene sets, we found high miR-106b and high miR-93 to significantly correlate with stem cell related gene sets[28] as well as EMT enriched gene sets,[29] further supporting an important role for these miRNAs in mediating stem cell and EMT-like processes in human breast cancer (Supplementary Figure 12).

DISCUSSION

In the present study, we identify a cluster of miRNA previously shown to overcome TGF-β-mediated growth inhibition,[11] the miR-106b-25 cluster, as a target of Six1. The data herein not only provide a mechanism for how Six1 may silence TGF-β-mediated growth inhibition in breast cancers, but also for how Six1 activates the TGF-β pathway. This is the first demonstration that the same miRNA cluster that overcomes TGF-β-mediated growth suppression can in fact also *promote* TGF-β signaling.

Indeed, we show that miR-106b-25 miRNAs can target the TGF-β inhibitor Smad7, and that upregulation of miR-106b-25 leads to an increase in TβRI. This increase in TβRI likely occurs, at least in part, due to Smad7 downregulation, as Smad7 is known to mediate degradation of the TβRI protein.[19] Recently, we demonstrated that upregulation of TβRI protein is necessary and sufficient for TGF-β activation and the induction of EMT downstream of Six1 in MCF7 cells.[9] Consistent with our previous data demonstrating that TβRI overexpression is sufficient for TGF-β pathway activation, we also observe an activation of this pathway with overexpression of the miR-106b-25 miRNAs alone as evidenced by an increase in transcriptional targets of the TGF-β signaling pathway (Figure 4e).

Several lines of evidence have demonstrated that the miR-106b-25 cluster and its individual miRNAs have pro-oncogenic functions, including mediating pro-proliferative and anti-apoptotic phenotypes.[12,13] Our results in this study expand the oncogenic potential of this miRNA cluster by demonstrating for the first time that these miRNA can also induce properties of EMT and TIC characteristics. The EMT changes induced by these miRNA are consistent with the oncogenic EMT phenotype induced by Six1 in MCF7 cells (Figure 5), suggesting that overexpression of the miR-106b-25 miRNAs may partly contribute to the induction of EMT downstream of Six1.

It is well recognized that the induction of EMT leads to an increase in stem/progenitor cell properties.[23] Indeed, Six1 transgenic mice whose mammary tumors display features of EMT, also demonstrate an increase in the stem/progenitor cell population.[8] Increased expression of the miR-106b-25 miRNAs in the mammary glands of Six1 transgenic mice suggests a possible role for these miRNA in regulation of the stem/progenitor pool (Figure 2c). Our results show for the first time that the miR-106b-25 miRNAs are sufficient to increase TIC capacity, and that they are required for the ability of Six1 to induce TIC characteristics *in vivo*. Interestingly, many recent studies have implicated the miR-106b-25 cluster in stem/progenitor cell biology.[14,30,31] Of interest, it was recently demonstrated that miR-106b and miR-93, along with iPSC transcription factors, can enhance reprogramming of somatic cells into induced pluripotent stem cells.[21] The iPSC phenotype, however, is dependent on downregulation of TGF-β signaling, where miR-106b and miR-93 target TβRII (in a mouse embryonic fibroblast context), while the TIC phenotype is known to be associated with an *upregulation* of TGF-β signaling.[23] In this study, we did not observe a downregulation of TβRII in response to Six1 or miR-106b-25 overexpression in MCF7 cells (Supplementary Figure 6).[9] However, interestingly, both the iPSC data and our data suggest a role for the miR-106b-25 cluster in the induction of stem cell properties, possibly through their ability to regulate TGF-β signaling in a context dependent, and seemingly opposite, manner.

As the increase of TIC capacity is known to be associated with tumor recurrence,[32] our data are further strengthened by our analysis of miRNA microarray data, which demonstrate that high miR-106b and high miR-93 expression significantly correlate with a shortened time to relapse in human breast cancer patients (Figure 7d). Of note, consistent with miRNA-induced iPSC data,[21] our data also suggests that miR-106b and miR-93 (which are in the same family of miRNA) are the primary inducers of the TIC phenotype as there is no difference in tumor recurrence when only miR-25 is highly expressed (Supplementary Figure 11). Similarly, in breast tumors where both miR-106b and miR-93 are highly expressed together, an enrichment in stem cell and *EMT* gene signatures can be observed (Supplementary Figure 12), further strengthening the argument that the miR-106b family of miRNAs may be important regulators of EMT and TIC phenotypes in human breast cancer.

Lastly, our data also demonstrate a significant correlation between miR-106b, Six1 and activated TGF-β signaling (nuclear Smad3) in human breast cancers. Critically, we show that tumors that express both high Six1 and high miR-106b have the highest percentage of activated TGF-β signaling (64.7%) (Figure 7). Of note, our data also show that in the presence of high Six1 and low miR-106b expression, 23.5% of tumors have activated TGF-β signaling, as opposed to 5.9% in tumors with low levels of Six1. These data suggest again that Six1 may activate TGF-β signaling through multiple mechanisms, however, the marked increase in TGF-β signaling when Six1 and miR-106b are both highly expressed strongly suggests that the Six1/miR-106b-25 axis is critical for activation of TGF-β signaling in human breast cancer.

In closing, these data have important ramifications for breast cancer treatment. Due to the traditional difficulties in targeting transcription factors, such as Six1, and the increasing promise for miRNAs as therapeutic targets,[33] the miR-106b-25 cluster could prove to be an effective target in cancers that express high levels of Six1. Furthermore, as TGF-β signaling can be tumor suppressive or tumor promotional, depending on context, one of the greatest concerns surrounding the use of TGF-β inhibitors in cancer is how to predict which patients will benefit in clinical trials. Our studies suggest a mechanism that we hypothesize provides a novel molecular explanation for the TGF-β paradox in breast cancers. Namely, examining breast tumors for Six1 and/or miR-106b-25 expression may ultimately provide a means to distinguish patients

likely to benefit from TGF-β inhibitors from those who may actually be harmed by such treatments.

MATERIALS AND METHODS

microRNA microarray and mRNA microarray

Total RNA preparations using TRIzol were sent to Thermo Scientific (Waltham, MA, USA) for miRNA microarray analysis. Three clonal isolates of MCF7-Ctrl and MCF7-Six1 RNA samples were submitted in replicates of four. miRNAs in which the difference between the groups (MCF7-Ctrl and MCF7-Six1) was statistically significant (P-value <0.05) were chosen for follow-up analysis. For mRNA microarray analysis, RNA was prepared in the same way as above, and submitted to the Genomics and Microarray Core at the University of Colorado Denver. The array was performed using the Affymetrix Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA). Analysis of microarray results have been described previously.[7]

Cell culture and constructs

Generation of MCF7-Ctrl and MCF7-Six1 cell lines was described previously.[34] The MCF7-Cluster cells were generated by inserting the cloned genomic region of the miR-106b-25 cluster into the miR-Express vector (Open Biosystems, Lafayette, CO, USA). EV and non-silencing (NS) miR-express constructs were obtained from open biosystems. The miRZip-cluster construct contains all three miRNA inhibitors (miRZip-106b, miRZip-93 and miRZip-25) in the same vector. MCF7-Ctrl and MCF7-Six1 cells were infected with miRZip lentivirus (System Biosciences, Mountain View, CA, USA). All MCF7 cells were selected with 2.5 μg/ml puromycin.

Real-time PCR

Total RNA was extracted with the miRNeasy RNA isolation kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol. For miRNA quantitative analysis, RNA was reverse transcribed using the miscript system (Qiagen), and qPCR was performed with miscript miRNA primers (Qiagen). All miRNA assays were done using ssoFast Evagreen supermix (Biorad, Hercules, CA, USA). For mRNA qPCR, cDNA synthesis was done with iscript (BioRad). Six1, Smad7 and PPIB primers were part of the gene expression assay collection (Applied Biosystems, Carlsbad, CA, USA). All qPCR was performed with the BioRad CFX96. Real-time PCR arrays were acquired from SABiosciences (Frederick, MD, USA) (RT^2 Profiler PCR array, PAHS-235, PAHS-405 and PAHS-090), and performed according to manufacturer instructions.

Western blot and immufluorescence

For western blots, whole cell lysates were isolated using RIPA buffer as previously described.[34] Antibodies used include: E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), β-catenin (BD Biosciences), TβRI (SCBT, Santa Cruz, CA, USA), TβRII (Cell Signaling, Danvers, MA, USA), p-Smad3 (Cell Signaling), total Smad3 (Invitrogen, Grand Island, NY, USA), Bim (Cell Signaling), p21 (Cell Signaling), β-Actin (sigma-aldrich, St Louis, MO, USA) and β-Tubulin (Invitrogen). Cell fractionation was performed as previously described.[35] Immunofluorescence was performed as previously described[7] with E-cadherin and β-catenin antibodies above.

Cell adhesion assay

Cells were plated in 96-well plates coated with Collagen I, Collagen IV, Laminin or Fibronectin (BD Biocoat, BD Biosciences), and assays were carried out as previously described.[7]

In-situ hybridization and Immunohistochemistry

Breast cancer tissue array (BRC711) was purchased from USA, Biomax Inc. (Rockville, MD, USA). *In situ* hybridization was performed with double DIG labeled miRNA LNA probes (Exiqon, Vedbaek, Denmark) using manufacturer's one-day protocol.[36] Modifications include 15 μg/ml Proteinase K for 8 min, overnight hybridization with 40 nM of LNA probe, and a formamide containing hybridization buffer (50% Formamide, 5 × SSC, 0.1% Tween, 50 μg/ml Heparin, 500 μg/ml yeast tRNA) at hybridization temperatures 30 degrees below the RNA Tm. Detection was achieved with BM purple (Roche, Basel, Switzerland) solution, and slides were counterstained in Nuclear Fast Red (Poly Scientific, Bay Shore, NY, USA). Slides were scored on a 0–4 scale with 4 representing the most intense staining. Scores were assigned independently by three individuals in a blinded manner and averaged. Serial sections of tumor arrays were previously stained and scored by a pathologist on a scale of 0–4 for nuclear Six1 (1:100; Atlas antibodies, Stockholm, Sweden) and nuclear Smad3 (5 μg/ml; Zymed, San Francisco, CA, USA), using immunohistochemistry protocols previously described.[7]

Luciferase assays

The 3′UTR of Smad7 was cloned into the psi-Check2 luciferase reporter (Promega, Madison, WI, USA). For perfect target luciferase assays, the exact complement sequence of each miRNA was also cloned into the psi-Check2 luciferase reporter. Constructs were transfected using Lipofectamine 2000 transfection reagent (Invitrogen), and at 48 h lysates were prepared and analyzed using the dual luciferase assay following the manufacturer's protocol (Promega). TOP-flash reporter assays were done as described previously[7] All luciferase assays were analyzed on the Modulus Microplate reader (Turner Biosystems, Madison, WI, USA).

TIC assays

Flow cytometry analysis and tumorshpere formation assays were performed as described previously[24] For *in vivo* tumor initiation assays, cells were counted and serially diluted in 100 μl of 1:1 PBS/Matrigel (no. 354234, BD Biosciences). Diluted cells were injected underneath the nipple of the number 4 mammary fat pad of 6-week-old female NOD/SCID mice. Tumor formation was monitored weekly by palpation. The frequency of tumorigenic cells was estimated at a setting of 95% confidence using the ELDA software application at http://bioinf.wehi.edu.au/software/elda/. All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

Analysis of miRNA microarray data

miRNA expression and clinical outcome data were acquired from a publically available dataset of 216 early-invasive primary breast cancers[27] (GSE22220). All samples were median-centered for each miRNA expression, and denoted high expression if above the median and low expression if below the median. Kaplan–Meier curves for tumors expressing both high miR-106b and high miR-93 were generated using WinStat for Excel (R Fitch Software, A-Prompt Corp, Whitehall, PA, USA). Gene set enrichment analysis methods are included in the Supplementary Information.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)